TRANSMETHYLATION AND METHIONINE BIOSYNTHESIS

Shapiro and Schlenk

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Preface

During the past decade it has been firmly established that S-adenosylmethionine is the most important methyl donor in transmethylation reactions. The development of methods for the biosynthesis of the sulfonium compound in large quantities has made it possible to study many new methyltransferases that are involved in a wide variety of metabolic pathways. These studies include such diverse fields as the biosynthesis of methionine, choline, cyclopropane fatty acids, sterols, and methylated nucleic acids. Since a conference on biological transmethylation or on methionine biosynthesis had not been held since the discovery of S-adenosylmethionine, it seemed appropriate to organize a symposium in which these topics would be considered in detail. The enthusiasm of the principal workers in these fields for such a conference led to the organization of a symposium on "Transmethylation and Methionine Biosynthesis." The Symposium was held on April 9 and 10, 1964, at Argonne National Laboratory, Argonne, Illinois, which is operated under the auspices of the United States Atomic Energy Commission. Because of the limitation of time, it was impossible to cover adequately all types of transmethylation reactions. Similarly, it was impossible to include in the program all investigators who have contributed to the areas discussed during the Symposium. However, we were fortunate to have many active workers in these fields participating in the conference. We were especially pleased that Professor Vincent du Vigneaud opened the Symposium with an historical survey of his classic contributions to the field of transmethylation.

The appearance of this publication so soon after the Symposium is due in large measure to the co-operation of the principal speakers and of the University of Chicago Press. The discussions were prepared from a stenographic transcript and a taped recording of the proceedings. In almost all cases, the discussants were given an opportunity to edit their remarks. However, the final compilation

of the discussion was made by the Editors who accept responsibility for any errors that may have been overlooked.

An extensive discussion took place during the Symposium concerning the use of abbreviations for S-adenosylmethionine and related sulfonium compounds. However, it was agreed that a satisfactory solution to this problem would be best achieved through the action of an official group dealing with nomenclature. In this volume the use of abbreviations for these compounds has been left to the discretion of the individual contributors.

The Symposium was sponsored by the Division of Biological and Medical Research, and we wish to express our thanks to Dr. Max R. Zelle, Director, for his encouragement and co-operation. We would also like to thank Dr. A. V. Crewe, Director of Argonne National Laboratory, who opened the Symposium, and Drs. D. D. Woods, G. L. Cantoni, J. R. Totter, and J. M. Buchanan, who served as the chairmen of the four scientific sessions. In behalf of all participants in the Symposium, we wish to express our appreciation to the many persons of Argonne National Laboratory who helped make the Symposium a pleasant and scientifically rewarding conference.

STANLEY K. SHAPIRO
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Contents

The Concept of Transmethylation in Mammalian Metabolism and Its Establishment by Isotopic Labeling through "In Vivo" Experimentation

Vincent du Vigneaud and Julian R. Rachele

It was just twenty-five years ago that the discovery of a metabolic relationship between choline, homocystine, and methionine was announced at the *Federation Meetings* in April, 1939, at Toronto (1). It was found that choline plus homocystine could replace methionine in the diet. This discovery, arising from a nutritional experiment, led eventually to the vast amount of experimental work in our own and other laboratories which culminated in the establishment of transmethylation as a metabolic process and set the stage for studies on the neogenesis of methyl groups.

This nutritional experiment was carried out in the fall of 1938 at Cornell University Medical College in collaboration with Chandler, Moyer, and Keppel (1). It was a continuation of earlier experiments at George Washington University Medical School in which the senior author in collaboration with Dyer and Kies (2) had been studying the mechanism of the conversion of homocystine to cystine in the rat and had designed an experiment employing an amino acid diet patterned after that of Rose and co-workers (3), which was based on the composition of casein but free of methionine and cystine. The composition of this diet is shown in Table I.

In order to have as little extraneous sulfur as possible in the vitamin supplement, the crystalline vitamins then available, namely, thiamine, riboflavin, and nicotinic acid, were used and a small amount of Ryzamin B (Burroughs Wellcome and Co., Inc.), an extract of rice polishings, was added as a source of other B factors that had not yet been isolated or even recognized. With this

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vitamin supplement it was found that the animals grew when methionine was added to the basal diet at a level of 1.4%, but when the methionine was replaced with homocystine at the equimolar level of 1.25%, no growth occurred. It was also found that the livers of the animals on the homocystine diet appeared yellowish at autopsy, and histological examination indicated fatty infiltration of the liver.

In the continuation of the investigation after transfer of the work from Washington to New York, it was decided as a first step to repeat exactly the Washington experiment, just to see if the same results would be obtained. The autopsy

TABLE I

COMPOSITION OF DIET

Amino acid mixture	23.6 gm
Dextrin	24.4 gm
Sucrose	15.0 gm
Salt mixture	4.0 gm
Agar	2.0 gm
Cod liver oil	5.0 gm
Lard	26.0 gm

AMINO ACID MIXTURE

Glycine	0.1 gm	L-Aspartic acid	0.2 gm
DL-Alanine	0.4 gm	DL-Serine	0.2 gm
DL-Valine	2.0 gm	L-Tyrosine	1.0 gm
DL-Leucine	2.6 gm	L-Histidine·HCl·H_2O	0.7 gm
DL-Isoleucine	1.8 gm	L-Arginine·HCl	0.6 gm
L-Proline	0.2 gm	DL-Lysine·2 HCl	4.5 gm
L-Hydroxyproline	0.1 gm	L-Tryptophan	0.4 gm
DL-Phenylalanine	1.5 gm	DL-Threonine	1.4 gm
L-Glutamic acid	2.0 gm	Sodium bicarbonate	3.9 gm

VITAMIN SUPPLEMENT[a]

Thiamine chloride	0.01 mg
Riboflavin	0.01 mg
Nicotinic acid	0.01 mg
Ryzamin B	12.5 mg
Dextrin	150. mg

[a] Supplied by two pills daily, each having the above composition.

results in the Washington work were fresh in mind, and it was known from the work of Best and co-workers (4) that a choline-free diet led to fatty infiltration of the liver. Since it was realized that the diets must have been practically choline-free, it was decided to add choline to the homocystine diet of one of the animals in the very first litter of rats employed, purely from the standpoint of preventing fatty liver. When the animals were weighed on the day after they were put on the diets, a startling fact was encountered. The animal receiving the homocystine diet plus choline had gained 4 gm overnight, whereas the animals receiving the unsupplemented homocystine diet had not gained weight (1, 5). This unexpected finding immediately gave rise to the thought that a

methyl group was being transferred from choline to the sulfur of homocysteine to give methionine, the homocysteine arising from the homocystine in the diet.

The concept of the transfer of a methyl group *in toto*, later termed *transmethylation* by this laboratory, was a new thought to us. However, such ideas are often anticipated by earlier speculations or suggestions, and a search of the literature revealed that Hofmeister (6) in 1894 had wondered whether a methyl group of one compound might be utilized for methylation of another compound. In the ensuing years various theories for the mechanism of biological methylation were considered. In a report of their studies on the production of trimethylarsine in molds, Challenger and Higginbottom (7) in 1935 discussed some of these possibilities involving various potential sources of the methyl group. For example, they considered the possibility that some ingredient of the cell substance containing a methylated nitrogen atom might, under the special conditions obtaining in the cell, lose a methyl group which, if it were eliminated with a positive charge, could be easily coordinated by the unshared electrons of tervalent arsenic. They also considered acetic acid and formaldehyde as possible sources of the methyl group. It was impossible at that time to distinguish between the possibilities, and in 1937, in their study of the methylation of mercaptans by molds, Challenger and Rawlings (8) appeared to favor formaldehyde as a precursor of the methyl group.[1]

The investigation in our laboratory revealing the unexpected growth on the homocystine diet containing choline was of course continued, and the chart in Fig. 1 shows the growth curves of some of the animals. It is evident that whenever choline was added to the diet the animals were able to grow with homocystine in place of methionine. It was also found that betaine, like choline, would support growth under these dietary conditions (5). Of a great number of compounds that were tested in later work in our laboratory, dimethylthetin (10), dimethylpropiothetin (11), and the dimethylethyl analogue of choline (12) were the only compounds that were effective in supporting growth on this diet. The terms "methyl donor" and "labile methyl compound" were used to indicate a compound which, when present in the diet, can support growth by making methyl groups available to the body. The term "labile methyl group" indicated the type of methyl group that could be so utilized. These were nutritional definitions and had no reference at that time to the actual mechanism involved.

Plate 1 shows the appearance of an animal that had been on a methyl-free diet for a long period of time. When choline was fed to such animals, growth resumed and they were brought back to normal appearance. If allowed to continue on the homocystine diet in the absence of choline or of other methyl donors, most of the animals eventually died.

In the extension of the original homocystine-choline nutritional experiment

[1] The extensive work by Challenger and co-workers on the methylation of arsenic, selenium, and tellurium compounds by molds as well as the historical background of the work is presented in several reviews (9).

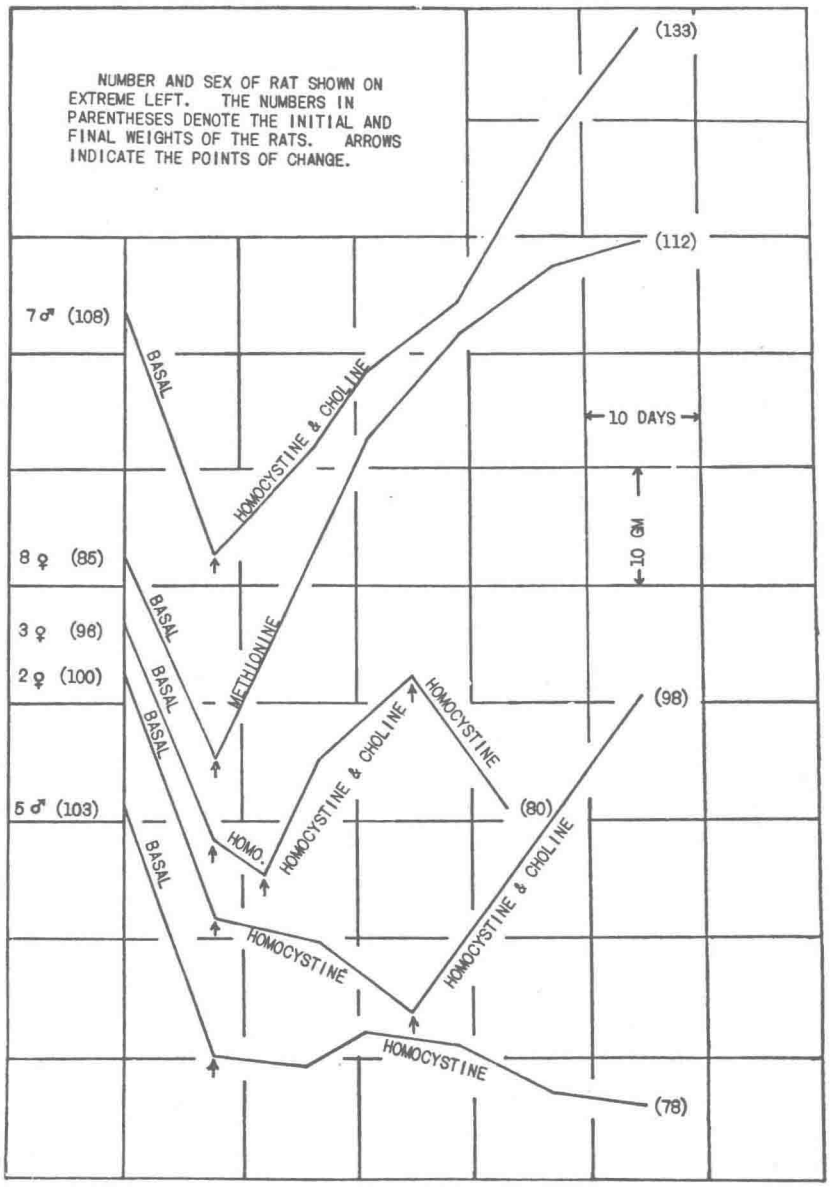

FIG. 1.—Growth of rats on a basal cystine-methionine-free amino acid diet (Table I) supplemented at indicated points with methionine, homocystine, or homocystine plus choline.

with several litters of rats it was noticed that occasionally an animal after losing some weight started to grow again on the homocystine diet without added choline as shown in Fig. 2. In the presentation of this work at the *Federation Meetings* in 1939, attention was called to this fact and the possible role of coprophagy was discussed. It was this occasional growth on a methyl-free diet that indicated that under certain conditions sufficient methyl groups were

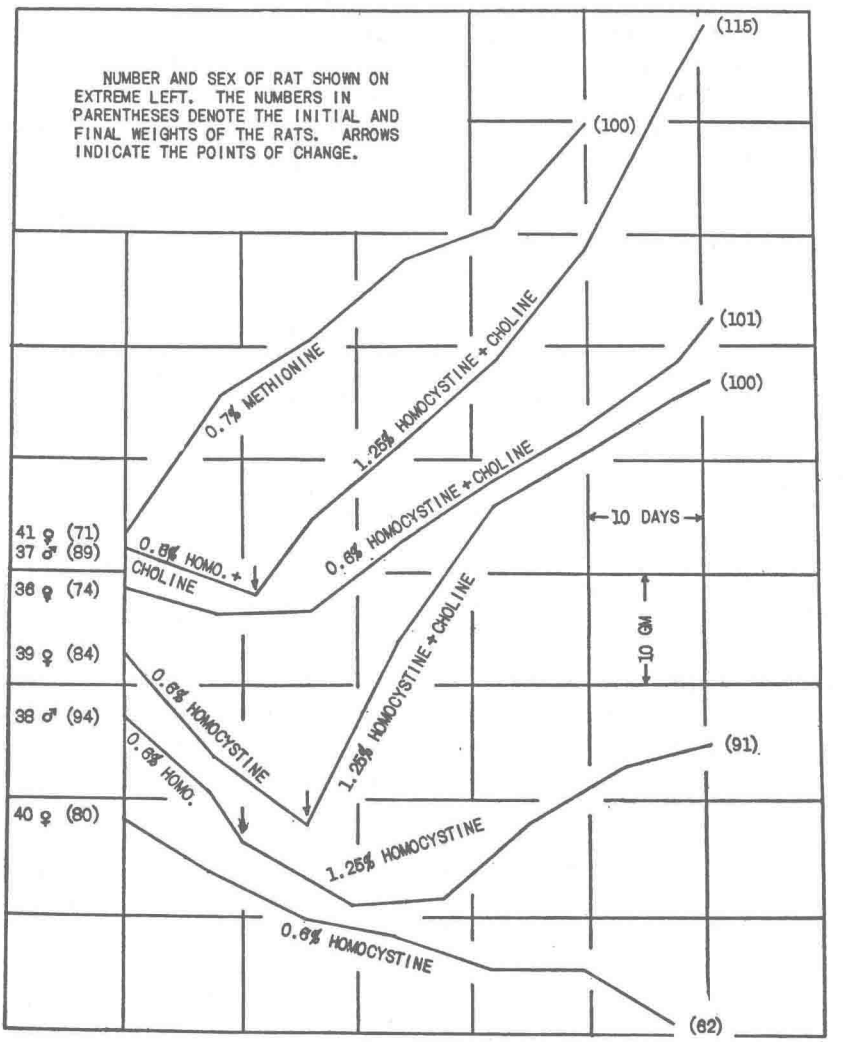

FIG. 2.—Growth of rats on a basal cystine-methionine-free amino acid diet (Table I) supplemented as indicated. The spontaneous growth of Rat No. 38 on a methyl-free homocystine-containing diet is to be noted.

synthesized to allow growth to take place. In fact, this observation led later to experiments in collaboration with Simmonds, Chandler, and Cohn (13) on animals with D_2O added to their drinking water. The appearance of deuterium in the methyl groups of tissue choline isolated from these animals (Table II) showed that biosynthesis of the methyl group had taken place. The question of whether this synthesis occurred in the tissues of the animal or in the intestinal tract was not settled experimentally for some years.

In 1949, an opportunity to obtain crucial information on this question was afforded by arrangement of a collaboration with Professor Reyniers and Dr. Luckey of LOBUND Research Institute at the University of Notre Dame involving the use of germ-free animals (14). The technique of detection of synthesis of methyl groups through the isolation of choline from rats whose body

TABLE II

DEUTERIUM CONTENTS OF BODY WATER AND CHOLINE METHYL GROUPS

RAT	AVERAGE DEUTERIUM IN BODY WATER (*A*) (atom %)	DEUTERIUM CONTENT			PER CENT OF CHOLINE METHYL DERIVED FROM BODY WATER (*B*/*A* × 100)
		Choline Chloroplatinate (atom %)	Trimethylamine Chloroplatinate (atom %)	Methyl Group of Choline (*B*) (atom %)	
102	3.1	0.60±0.01[a]	0.15±0.06 0.21±0.01[a]	0.24[b]	7.7[b]
103	3.2	0.39±0.06	0.23±0.08 0.25±0.02[a]	0.27[b]	8.5[b]

[a] Microdeuterium method with the mass spectrometer.

[b] Value calculated from the data obtained by the microdeuterium method because of the smaller error in analysis.

water contained D_2O was again utilized. Rats of the LOBUND strain were maintained with D_2O in their drinking water under both germ-free and non-sterile conditions at LOBUND, and for comparative purposes animals of the Rockland Farms strain were maintained at the Cornell Laboratory under the usual laboratory conditions on the same dietary regimen as that used at LOBUND. Isolation of the choline and creatine from the tissues of all the animals and determination of the deuterium content in the methyl groups were carried out at the Cornell Laboratory in collaboration with Ressler and Rachele (14). The data in Table III show the deuterium content of the methyl groups of choline from the germ-free and non-sterile rats, as well as the ratio of the deuterium content of the choline methyl group to that of the body water. Similar values were obtained for the isotopic content of the creatine methyl group. From these results it was obvious that methyl groups had been synthesized in the absence of intestinal flora, and thus this synthesis must have occurred in the tissues of the rat. Meanwhile, Sakami and Welch (15) demon-

strated that methyl biosynthesis could take place *in vivo* and *in vitro* by the use of C^{14}-labeled acetone, formate, and formaldehyde. In the light of what is now known concerning the relationship of vitamin B_{12} and folic acid to the *de novo* synthesis of methyl groups as a result of the pioneering work of Toennies, Bennett, Medes, Stekol, and co-workers (16), it would still seem that intestinal synthesis may have been involved when growth was observed on the methyl-free, homocystine-containing diet; however, instead of the synthesis of methyl

TABLE III

DEUTERIUM CONTENT OF METHYL GROUPS OF CHOLINE FROM GERM-FREE AND NON-STERILE ANIMALS

RAT	EXPERIMENTAL PERIOD (days)	DEUTERIUM CONTENT			$B/A \times 100$
		Body Water (average) (*A*) (atom % excess)	Trimethylamine Chloroplatinate (atom % excess)	Methyl Groups of Choline (*B*) (atom % excess)	
GERM-FREE ANIMALS					
LOBUND III	23	2.44	0.14	0.16	6.4
LOBUND IV	10	2.99	0.09	0.10	3.3
NON-STERILE ANIMALS AT LOBUND					
LOBUND I	21	2.20	0.19	0.21	9.6
LOBUND II	21	2.27	0.18	0.20	8.8
NON-STERILE ANIMALS AT CORNELL					
CUMC 710[a]	21	3.29	0.23	0.26	7.8
CUMC 707	21	3.08	0.19	0.21	6.9
CUMC 708	21	2.52	0.19	0.21	8.4
CUMC 709	21	3.01	0.21	0.23	7.8

[a] Cornell University Medical College strain.

groups having taken place by the intestinal flora, it would now appear to have been the synthesis of folic acid and B_{12} in the intestinal tract, which then enabled the animals to synthesize sufficient methyl groups in the tissues.

In the 1939 *Federation* presentation in which the suggestion was made that a methyl group was transferred *in toto* from choline to homocystine to form methionine, it was also suggested that the reaction might be metabolically reversible and that the methyl group of methionine might be used for the synthesis of choline by transmethylation. If this were true, it would account for the findings of Eckstein and co-workers (17) and Channon and co-workers (18) that methionine as well as choline was lipotropic, whereas homocystine was not.

These suggestions were enthusiastically received at Toronto, but in the course of time considerable skepticism developed regarding the concept of transmethylation. It was realized that this concept must in some way be subjected to direct experimental test. In seeking such a test, it was decided to label the methyl group with deuterium and see if its metabolism could be traced in that way.

In collaboration with Chandler, Cohn, and Brown (19) methionine was synthesized with the methyl group labeled with deuterium according to the method outlined in Fig. 3 and fed to rats on the amino acid diet described in Table I. Short-term experiments showed that the deuteriomethyl group appeared in quite high amounts in the methyl groups of choline and creatine. Thus the carbon of the methyl group could be traced metabolically by labeling it with deuterium. However, there remained the question of whether the methyl

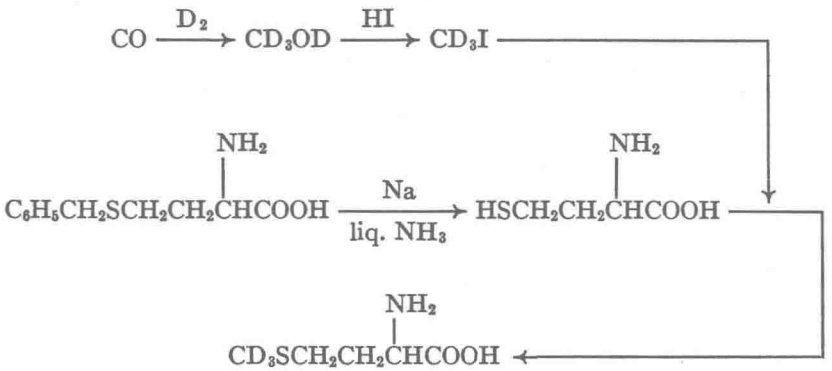

FIG. 3.—Synthesis of methionine labeled in the methyl group with deuterium.

group was really being transferred *in toto* or was being utilized by oxidation and subsequent reduction through an intermediate such as formaldehyde. If formaldehyde were involved, it would mean a loss of one of the three deuteriums in the methyl group, since on regeneration of the methyl group from formaldehyde only two deuteriums of the original methyl group could remain attached to the carbon. It is obvious that, no matter how long the deuteriomethionine were fed, if formaldehyde were the obligatory intermediate of the three methyl groups of the body choline and the methyl group of creatine, their deuterium content could not exceed 66.7% of the deuterium content of the ingested methionine.

To obtain information pertinent to this question long-term experiments were carried out in collaboration with Cohn, Chandler, Schenck, and Simmonds (20). A chart of two such long-term experiments in which the deuteriomethionine was fed for 54 and 94 days is shown in Fig. 4. The curves reflect the increase in deuterium content of the creatinine isolated from the urine during the course of the experiments and also the deuterium content of the choline and creatine

of the tissues at the end of the experiments. The ordinate represents the percentage of the deuterium in the methyl groups of the isolated compounds derived from the deuteriomethyl group of the ingested methionine. It may be noted that these percentages exceeded 85% after 94 days and that by the end of the experimental period the creatinine in the urine had derived approximately the same percentage of deuteriomethyl groups from the deuteriomethionine of the diet as had the choline and creatine of the tissues. Table IV shows the actual data on the deuterium contents of the methyl group of tissue choline and creatine in experiments of 3- to 94-day duration. The absolute deuterium

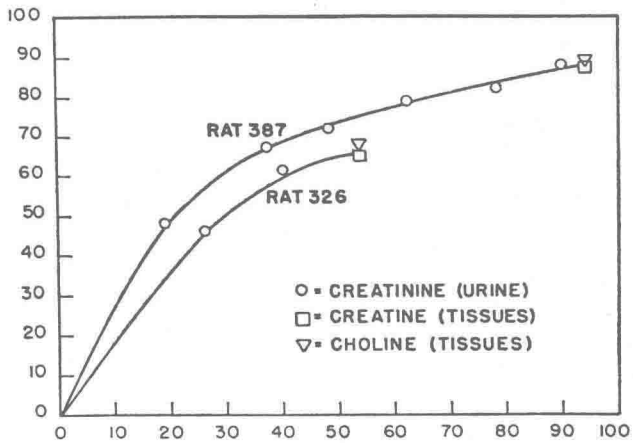

FIG. 4.—Incorporation of deuterium into the methyl groups of compounds isolated from the tissues and urine of rats fed methionine labeled in the methyl group with deuterium. The abscissa represents the time in days and the ordinate represents 100 times the ratio of the atom % deuterium in the methyl group of the isolated compounds to the atom % deuterium in the methyl group of the administered methionine. The percentages for the urinary creatinines are plotted at the mid-points of the time intervals during which the urines were collected.

contents of approximately 74 and 73 atom % for the methyl groups of choline and creatine, respectively, in the 94-day experiment represent 89 and 87% of the deuterium content of the methyl group of the administered methionine. This high degree of incorporation of the deuterium of the methyl group of methionine offered convincing evidence that the methyl group could be transferred as a unit and that it was thus utilized via transmethylation for the synthesis of choline and creatine *in vivo*. If formaldehyde were an obligatory intermediate, the absolute deuterium content of the choline and creatine methyl groups could not have exceeded 66.7 atom % even if during metabolism the trideuteriomethyl-labeled methionine species had accumulated in the tissue protein to a hypothetical level of 100%. Furthermore, the fact that the absolute deuterium contents of the methyl groups of choline and creatine were nearly identical

indicated strongly that all three methyl groups of choline came from the same precursor as the methyl group of creatine. If two of the methyl groups had come from formaldehyde and one by transmethylation, then the maximum atom per cent of deuterium in the methyl groups of choline would have been 7/9 of the creatine figure or 57 atom %. Moreover, if there were some dilution from other sources of formaldehyde or of one-carbon fragments involved in the synthesis of these methyl groups, this figure would of course have been even lower than 57 atom %.

This long-term feeding experiment with deuteriomethyl-labeled methionine demonstrated the reality of the concept of transmethylation from methionine to choline and creatine. To find out whether the methyl group of choline could

TABLE IV

INCORPORATION OF DEUTERIUM OF THE METHYL GROUP OF DIETARY METHIONINE INTO THE METHYL GROUPS OF TISSUE CHOLINE AND CREATINE

Rat	Experimental Period (days)	Deuterium in Methyl Group of Ingested Methionine (*A*) (atom %)	Choline[a]		Creatine[b]	
			Deuterium in Methyl Group (*B*) (atom %)	*B*/*A* ×100	Deuterium in Methyl Group (*C*) (atom %)	*C*/*A* ×100
325	3	87.5	11.9	13.7	7.9	9.1
226	23	87.5	50.2	57.4		
326	54	87.5	59.0	67.3	56.7	64.8
387	94	83.6	74.2	88.6	73.0	87.3
					72.8	87.1

[a] Analyzed as choline chloroplatinate.

[b] Analyzed as creatinine zinc chloride, except for the last value which was obtained from the analysis of creatinine potassium picrate.

be traced to methionine, choline with deuterium-labeled methyl groups was synthesized and fed to animals on a methionine-free diet containing homocystine as the only sulfur-containing amino acid (21). The methyl group of the methionine subsequently isolated from the body tissues did in fact contain a considerable concentration of deuterium. Furthermore, the incorporation of deuterium into the methyl group of tissue methionine was found to occur even when ordinary methionine was fed along with the deuteriocholine.

Subsequent experimentation in our own and other laboratories, in which various compounds were studied with regard to transmethylation *in vivo* and *in vitro*, enabled a correlation to be made in 1950 of the various hypotheses into the schematic diagram shown in Fig. 5.[2]

By studying betaine labeled with both deuterium and N^{15}, it was shown that

[2] For the detailed discussion and development of these metabolic interrelationships, see *A trail of research in sulfur chemistry and metabolism and related fields*, V. du Vigneaud (22).